

AD _____

Award Number: DAMD17-01-1-0340

TITLE: Epstein Barr Virus and Breast Cancer

PRINCIPAL INVESTIGATOR: Wing C. Chan, M.D.
Kishor G. Bhatia, Ph.D.

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, NE 68198-6810

REPORT DATE: September 2005

TYPE OF REPORT: Final

20060215 172

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE 01-09-2005		2. REPORT TYPE Final		3. DATES COVERED 1 Sep 2001 –31 Aug 2005	
4. TITLE AND SUBTITLE Epstein Barr Virus and Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-01-1-0340	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Wing C. Chan, M.D. Kishor G. Bhatia, Ph.D.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Nebraska Medical Center Omaha, NE 68198-6810				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT <p>Studies on the association of Epstein-Barr virus with Breast Carcinoma have reported conflicting results. They have in fact raised questions about a geographic variance in this association and the technical challenges of localizing the virus in tissue. We have addressed both these questions in our study. To look for a geographic variance, we analyzed breast carcinoma cases from four countries: USA (n=100), India (n=100), China (n=79) and Saudi Arabia (n=23). Each case was subjected to Conventional and Real-Time PCR with EBNA-1. PCR positive cases were further analyzed by EBER in-situ hybridization to localize the virus and a few cases were studied by Laser Microdissection (LMD). Our overall PCR positivity rate was 35-55% and did not exhibit any significant geographic variance. EBER in-situ confirmed the presence of the virus in the tumor cells in only four cases. The remaining cases were harboring the virus in the lymphoid cells infiltrating the tumor. LMD showed the presence of EBV in EBER positive as well as some negative cases (all PCR positive cases) but EBV could also be demonstrated in the fibrous tissue. Considering the entire spectrum of results obtained in this study we conclude that EBV does not play any major role in the pathogenesis of breast carcinoma.</p>					
15. SUBJECT TERMS Epstein-Barr virus (EBV), Breast cancer pathogenesis					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 16	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	11
Reportable Outcomes.....	12
Conclusions.....	13
References.....	14
Appendices.....	15

INTRODUCTION

Several studies have been done to explore a relationship between Breast Cancer and Epstein Barr Virus (EBV), however, two major issues still need to be addressed:

- 1) Geographic variability (as seen in other malignancies associated with EBV), and
- 2) Technical challenges in detecting EBV within the tumor cells.

We have evaluated both issues in our study. With most studies reporting a negative correlation between breast cancer and EBV (1-7), our aim was to look for geographical variations in this association. Therefore, we have studied samples from 4 countries, USA, China, India and Saudi Arabia. It has also been established that different assays used on the same sample yield different results in the detection of EBV (8). EBER in-situ hybridization, considered as the "gold standard" for EBV detection in other EBV related tumors, may not give definitive results for EBV detection in breast cancer tumor cells. Several other commonly used methods, such as Southern blot hybridization and immunohistochemistry all have limitations. (12,13). Therefore, we have used a combined approach and subjected each sample to a routine PCR screening for EBNA-1, followed by a real-time quantitative PCR which allows measurement of the amount/copy number of viral DNA. PCR positive cases were then subjected to EBER in-situ hybridization (ISH) to localize the EBV and a few cases were then selected for laser microdissection (LMD). This was in accordance with a recent National Cancer Institute recommendation of combining real time quantitative PCR with Laser Microdissection to improve localization of viral DNA to benign or malignant components of a tissue sample (8).

BODY

We have analyzed a total of 302 cases from 4 different geographical areas, including 100 cases each from the USA and India, 79 cases from China and 23 cases from Saudi Arabia. All the samples were screened for the presence of EBV genome using primers flanking the EBV nuclear antigen 1 (EBNA-1) region. Real-time PCR was also performed using the same primer on the American, Chinese and Saudi Arabia cases. Real-time PCR was not carried out on the Indian cases (the last sample group to be studied) because our data did not show that real-time PCR has advantage on sensitivity compared with conventional PCR. PCR for the detection of the defective heterogeneous EBV was also performed on all these cohorts.

EBER in-situ hybridization was performed on all the PCR positive cases to determine whether the EBV expression occurs in the tumor compartment or in the infiltrating lymphocytes.

PCR RESULTS

We have an overall PCR positivity rate of 34-57% using the EBNA-1 primer.

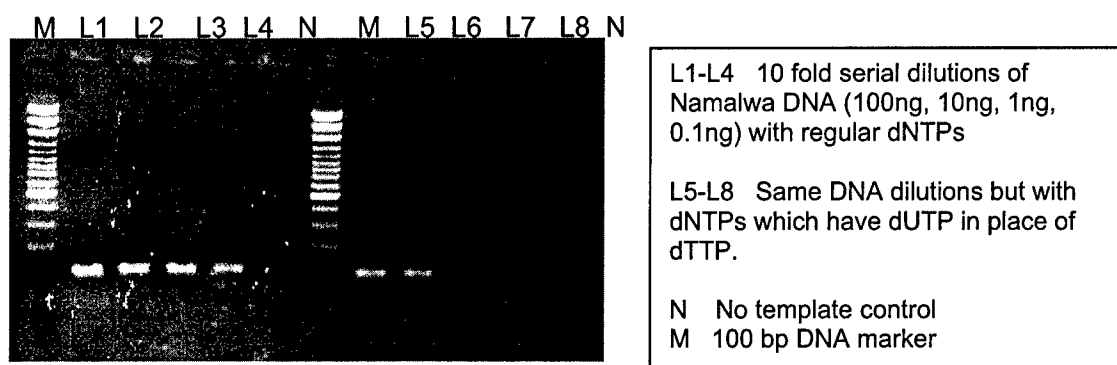
TABLE 1.

	CONVENTIONAL PCR	REAL-TIME PCR
AMERICAN CASES	45/100 (45%)	7/100 (7%)
CHINESE CASES	45/79 (57%)	39/79 (49.4%)
SAUDI ARABIA CASES	13/23 (56.5%)	5/23 (21.7%)
INDIAN CASES	34/100 (34%)	Not done

However, in a PCR reaction this positive signal could be generated by the presence of EBV in any component of the tissue. Therefore, we performed EBER in-situ hybridization on the PCR positive cases to localize the positive signal. It has been reported by earlier studies that this PCR positivity is more often from the lymphoid cells infiltrating the tumor than from the tumor cells themselves.

Also, as seen in Table 1, we had a discrepancy in results obtained by using the 2 different PCR assays, conventional and real time PCR, on the same samples. We investigated this further and came to the conclusion that the dUTP used in the dNTP mixture in the Taqman Universal Master Mix decreases the efficiency of the reaction. This is demonstrated in Figure 1. Here we set up 2 identical sets of PCR reactions. All the conditions and reagents were the same in both the reactions except for the dNTPs. The first set had the regular dNTP mixture (dATP, dGTP, dCTP, dTTP) while the second set had the dNTP mixture with dUTP in place of the dTTP. Both the sets were run in the same PCR machine at the same time so that all the PCR conditions were the same. 10 fold serial dilutions of the EBV positive Namalwa DNA were used as the positive controls.

FIGURE 1.



As seen in the above figure, the PCR product obtained using the dUTP (L5-L8) is significantly less as compared to that using the regular dNTPs (L1-L4). This finding suggests that conventional PCR is more suitable for our study where we are dealing with very low copy numbers of EBV in our samples. Therefore, we decided to perform only the conventional PCR on the Indian cases.

PCR performed for detection of the Heterogeneous EBV, showed a positivity rate of 1-13%. Therefore, we concluded that this form of EBV does not seem to play an important role in the development of Breast Carcinoma.

EBER IN-SITU HYBRIDISATION RESULTS

EBER in-situ hybridization was performed on all the PCR positive cases.

TABLE 2.

	AMERICAN CASES	CHINESE CASES	INDIAN CASES	SAUDI ARABIA CASES
EBER +VE [TOTAL NO. OF CASES]	12/45 (26.7%)	26/45 (57.8%)	13/34 (38.2%)	0/13
TUMOR CELLS +VE	4/12	0/26	0/13	-
LYMPHOID CELLS +VE	7/12	26/26	13/13	-

As can be seen in Table 2, the majority of the cases that were PCR positive were harboring the EBV in the lymphoid cells infiltrating the tumor, and not in the tumor cells. In our entire study we had only 4 cases (all from the American group) that showed the presence of the virus in the tumor cells. Of these 4 cases, only 1 case (US39) had 40-50% of the tumor cells positive for EBV (Figure 2). The remaining 3 cases had only occasional tumor cells positive by EBER. The Chinese, Indian and Saudi Arabian sample groups did not have any cases showing positive staining for EBER in the tumor cells. This finding reinforces previous reports indicating that EBV infection of the breast carcinoma cells is a very uncommon event. Even for positive cases, breast cancer is unlike "traditional" EBV-related tumors, which express EBER in virtually all tumor cells (8).

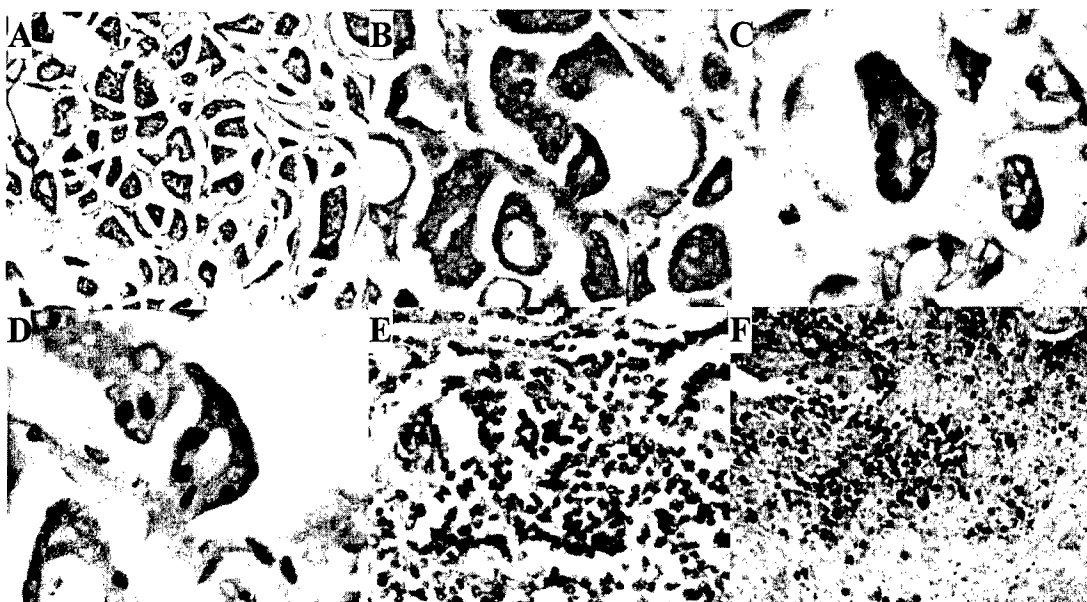


Figure 2. EBER-1 ISH in positive control and breast cancer sections. A, US case #39 shows nuclear staining of tumor cells (10X). B-D, same sections shown in higher power view. E shows positive staining of infiltrating lymphocytes in the tumor stroma, and F, positive control staining on a known EBV positive case.

However, we did make some incidental interesting findings while performing the in-situ hybridization studies. We demonstrated in rare cases, positive staining for EBER in endothelial cells, stromal cells and surrounding normal breast glands and in 1 case, in the normal skin epidermal cells over the breast tissue (Figure 3). This suggests that EBV can infect a broader spectrum of cells than previously recognized.

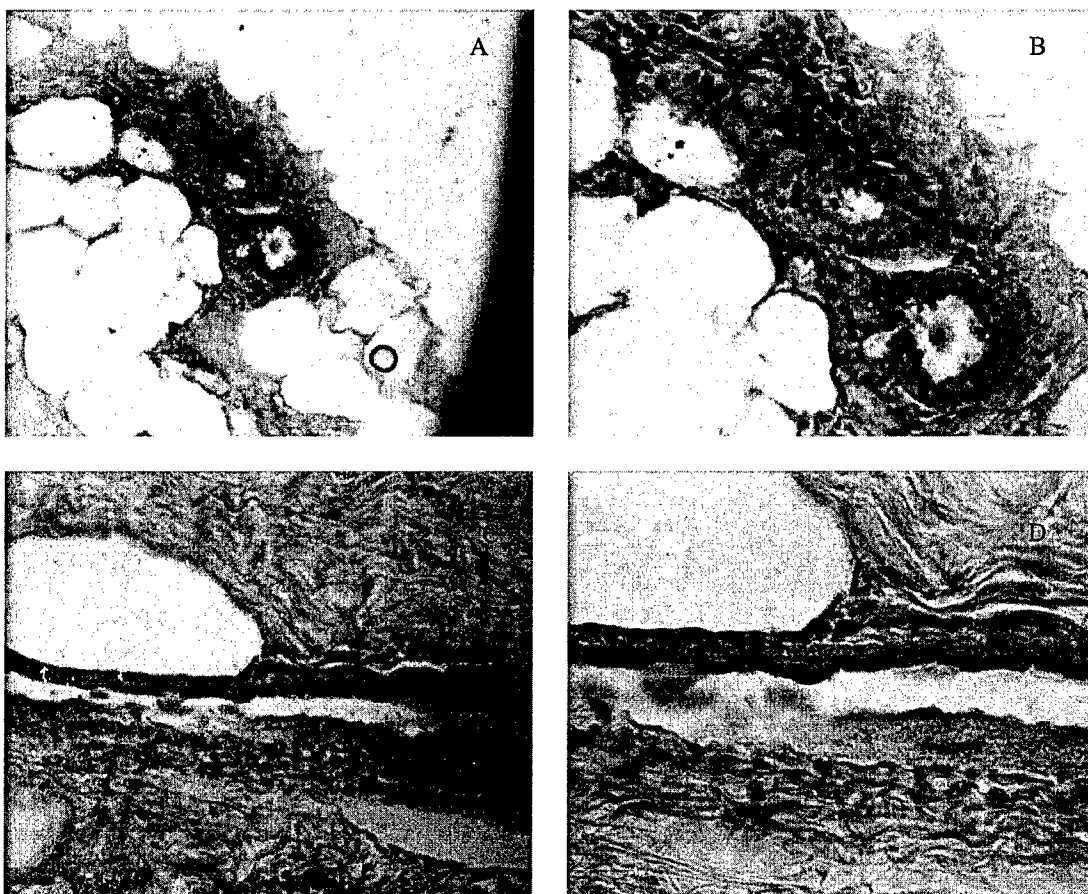


Figure 3. EBER ISH detected positive staining (dark blue cells) in some normal glandular cells in Panel A (low magnification) and Panel B (same section shown in higher magnification). In one case, some endothelial cells are positiv, Panel C (low power) and Panel D (high power).

LASER MICRODISSECTION

Previous studies suggest that breast tumors may express EBERs less abundantly than other malignancies (9,10) or have a previously unrecognized form of EBV infection characterized by EBER down regulation (2,11). Laser microdissection was performed on 2 groups of samples [as described in the Annual Report for 2004]. Samples that were PCR positive and EBER ISH positive in tumor cells and/or lymphocytes (group 1) vs PCR positive but EBER negative cases (group 2). PCR amplifying EBNA-1 on the microdissected cells demonstrated high EBV positivity (2/5 in group 1 and 4/8 in group 2) in the tumor cells irrespective of the EBER ISH status.

Another interesting finding that we made on microdissection was that our acellular fibrous controls which were used as negative controls, also showed positive results on PCR with EBNA1 (Figure 2A and B). The "no template" negative controls run with the same reaction were always negative, ruling out contamination during PCR as a possible explanation.

FIGURE 4

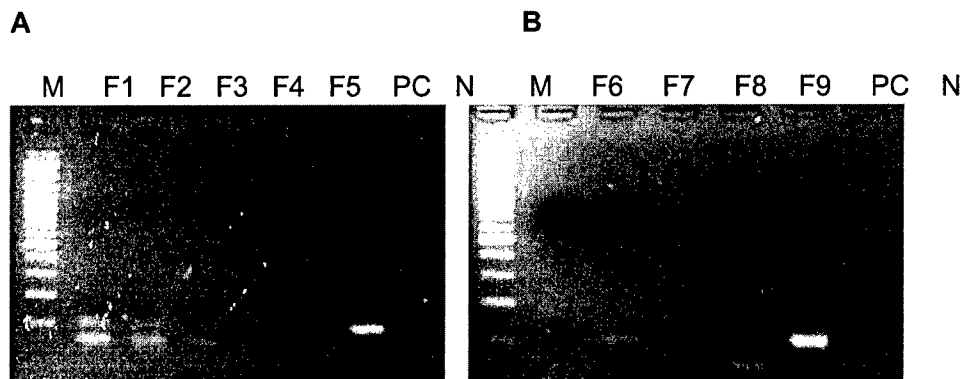


Fig 4. PCR for EBNA on microdissected fibrous tissue from 9 different samples (F1-F9).

PC: EBV positive Namalwa DNA used as a positive control

N: "No template" control

M: 100 bp DNA marker

As can be seen in figure4, 4 samples (F1, F2, F7, F9) showed positive bands for EBNA-1, while the "no template" control (N) run in the same reaction was negative.

In order to rule out the possibility of contamination during the laser microdissection procedure, we microdissected samples of the membrane (devoid of any tissue section) from the PEN membrane coated slides (Leica) used for microdissection. These were then subjected to a routine EBNA-1 PCR. There were no positive bands obtained.

These findings with the fibrous tissue suggest the possibility of "free virus" in the tissue and warrant further investigation. It also makes the interpretation of all microdissection studies difficult.

KEY RESEARCH ACCOMPLISHMENTS

- ❖ Established EBER in situ hybridization for localization of EBV infected cells, and found a wide spectrum of target cells for EBV.
- ❖ Studied cases from different geographical regions: 100 USA cases, 79 China cases, 23 Saudi Arabia cases and 40 of Indian cases of breast carcinoma for EBV by PCR and EBER ISH.
- ❖ Developed Real-Time PCR assay for the EBNA1 region and also an assay for the defective EBV.
- ❖ Integrated different assays (PCR, ISH, and microdissection) for the interpretation of data.
- ❖ Developed LMD techniques for the isolation of tumor cells and other cellular and non-cellular compartments in tissue sections.
- ❖ Concluded that EBV infection of breast cancer cells is rare and even in positive cases, it is a sporadic event. It is unlikely that EBV plays a significant role in the pathogenesis and progression of breast carcinoma.

REPORTABLE OUTCOMES:

An abstract titled " Epstein-Barr virus and breast cancer" was presented in the " Era of Hope – Department of Defense Breast Cancer Research Program meeting" on September 24-27, 2002 at Orlando, FL.

One technologist was trained for molecular biology and Laser microdissection techniques supported by this reward.

A manuscript is under preparation.

CONCLUSIONS

Our study showed an EBV positivity rate of around 35-55% by PCR on EBNA1 using whole tissue sections without significant geographic variance. PCR on microdissected tumor cells also detected virus in 40-50% of specimens of PCR positive samples irrespective of EBER status. However, the finding of microdissected acellular fibrous tissue giving positive results on EBV PCR raises a big concern about the specificity of findings on the tumor tissue. The data indicates that study on viral pathogenesis is more challenging than traditional methods suggested, especially with low viral load and multiple cell types that can harbor the virus. In conclusion, our data strongly argues against EBV playing a major role in breast cancer pathogenesis.

An interesting incident finding is that besides infiltrating lymphocytes, occasionally normal glandular cells and more often, endothelial cells were positive for EBV, suggesting that EBV may infect more cell types than previously suggested.

REFERENCES

1. Dadmanesh F, Peterse JL, Sapino A, Fonelli A, Eusebi V. Lymphoepithelioma-like carcinoma of the breast: lack of evidence of Epstein-Barr virus infection. *Histopathology* 2001; July; 38: 54-61.
2. Peiguo G, Chang KL, Chen YY, Chen WG, Weiss L. No significant association of Epstein-Barr virus infection with invasive breast carcinoma. *American Am J Pathol.* 2001; 159(2): 571-8.
3. McCall SA, Lichy JH, Bijwaard KE, Aguilera NS, Chu WS, Taubenberger JK. Epstein-Barr virus detection in ductal carcinoma of the breast. *Journal of National Cancer Institute* 2001; 93(2): 148-150.
4. Herrmann K, Niedobitek G. Lack of evidence for an association of Epstein-Barr virus infection with breast carcinoma. *Breast Cancer Res.* 2003;5(1):R13-7.
5. Despande CG, Badve S, Kidwai N, Longnecker R. Lack of expression of the Epstein-Barr Virus (EBV) gene products, EBERs, EBNA1, LMP1, and LMP2A in breast cancer cells. *Lab Invest.* 2002 Sep; 82(9): 1193-9.
6. Herrmann K, Niedobitek G. Epstein-Barr virus associated carcinomas: facts and fiction. *J Pathol* 2003; 199:140-5.
7. Perrigoue JG, Boon JA, Friedl A, Newton MA, Ahlquist P, Sugden B. Lack of association between EBV and Breast Carcinoma. *Cancer Epidemiol Biomarkers Prev* 2005; 14(4): 809-814.
8. Glaser SL, Hsu JL, Gulley ML. Epstein-Barr Virus and Breast Cancer: state of the evidence for viral carcinogenesis. *Cancer Epidemiol Biomarkers Prev* 2004; 13(5): 688-97.
9. Labrecque LG, Barnes DM, Fentiman IS, Griffin BE. Epstein-Barr virus in epithelial cell tumors: a breast cancer study. *Cancer Res* 1995; 55: 39-45.
10. Bonnet M, Guinebretiere JM, Kremmer E, et al. Detection of Epstein-Barr virus in invasive breast cancers. *J Natl Cancer Inst* 1999; 91: 1376-81.
11. Magrath I, Bhatia K. Breast cancer: a new Epstein barr virus associated disease? *J Natl Cancer Inst* 1999; 91: 1349-50.
12. Takeuchi H, Kobayashi R, Hasegawa M, and Hirai K. 1997. Detection of latent Epstein-Barr virus (EBV) DNA in paraffin sections of nasopharyngeal carcinomas expressing no EBV-encoded small RNAs using in situ PCR. *Arch Virol.* 1997; 142(9): 1743-56.
13. Herrmann K, Niedobitek G. Lack of evidence for an association of Epstein-Barr virus infection with breast carcinoma. *Breast Cancer Res.* 2003; 5(1): R13-7. Epub 2002 Dec 04.

A poster titled " Epstein-Barr virus and breast cancer" was presented in the " Era of Hope – Department of Defense Breast Cancer Research Program meeting" on September 24-27, 2002 at Orlando, FL.

EPSTEIN-BARR VIRUS AND BREAST CANCER

and Hong Kong Special Administrative Region, and Research Center, Tsinghua University, Beijing, China

Abstract

[illegible]

Approximately 10% of IPV with latent cytomegalovirus were also screened at the Naval Specialized Hospital, Naval Airfield, with 1051 human sera, are from two different seroprevalence surveys. 40% latent infection from Kibira Cancer Center, Pungwe, Zimbabwe, India and 6% samples from Kivumbi Cancer Centre. To determine the prevalence of CMV, amplification of the unique long (udl) and RL131 gene was carried out. Study 1 of 61 samples from Kivumbi were positive for CMV, whereas 14% of blood sera sampled from India from 1991 to 1995 were

The 100% claim is based on data from India and Mexico. But in the U.S. over 40 million live exports of *E. coli* to other individuals. All but 1 sample from the U.S. indicating that more than 99% people failed to consume *E. coli* as a toxin itself. Two U.S. samples did show staining of antibodies by *E. coli*. These data suggest that the antibodies and in the field of *E. coli*, associated with human cancers are heterogeneous, variable and there is a close similarity between the *E. coli* and *E. coli* 100% of the diseases may be due to the detection of *E. coli* by 99% as indicating *E. coli* itself. The more the probability of the presence of *E. coli*, defined *E. coli* as human cells that failed to express *E. coli* needs to be improved by the other studies.

Introduction

(Tyrone then wrote (1975)) is associated with a range of literary and dramatic writings. African American writers, such as Langston Hughes, Richard Wright, and Lorraine Hansberry, and white writers, such as Ralph Ellison, James Wright, and William Faulkner, all wrote about the blues.

breast cancer is the most frequent malignancy and the leading cause of cancer death among women in Western countries. Although the etiology of breast cancer is not completely understood, genetic background and hormonal effects are believed to play important roles in its development. In recent years, researchers have questioned whether FST may play a role in the development of breast cancer. However, a number of reports on this subject, with heterogeneous results, have been published. This would be related to the different samples employed and also possibly to geographic variations in the prevalence of the FST alleles.

The aims of this study are: 1) To compare different sources for the detection of EPR provide different subgroups; 2) To compare if there is a geographical variation in the distribution of EPR with known cases or samples obtained from the USA; 3) To determine if the presence of EPR in these samples is associated to factors (age and sex); 4) To assess the overall prevalence in the clinical environment of the source (cfr. 1); 5) To assess the overall prevalence within of the total source (cfr. 1) of patients breast with at least one metastasis.

Figura 1. Diagrama de flux privind procesul de evaluare a impactului asupra mediului în proiectarea și construirea unei noi stații de alimentare cu apă caldă în orașul Iași

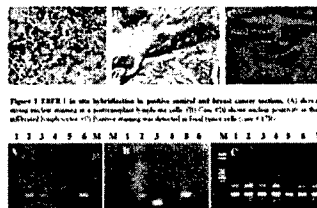


Figure 1. ERFR-1 *in situ* hybridization in positive control and breast cancer sections. (A) shows



Figure 1 PCR amplification of H2F1 (A) and H2F-1 (B) promoters (15) in 49 breast cancer samples (lanes 1-4 in both A and B). Lane 5 represents a sample which is $H2F-1$ positive for H2F1, but negative for H2F-1 (lane 6 (A) and lane 5 (B) are α -tubulin DNA; lane 7 (A) and lane 6 (B) are no template control). PCR breast cancer samples amplified with human α -globin (16) as housekeeping gene (17) are shown.

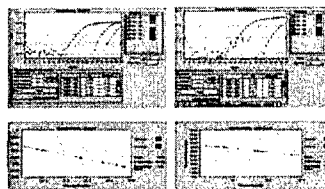


Figure 3. Amplification plot of real-time PCR of HBNA-1. (A) Serially diluted plasmid containing HBNA-1 (10¹–10⁷ copies per tube) were analysed for HBNA-1. (B) Standard curve generated from the mean value amplification measurements. Blank circles represent controls and circles are lowest control samples and open circles and filled circles amplified in duplicate. Type III DNA is normally 10⁴ copies of each sample. (C) Serially diluted HB-1 (10¹–10⁷ copies) by myeloma cells (see text). Arrow: 500 ng to 0.5 ng were analysed for HBNA-1.

TABLE 1. PCR PRIMER AND PROBES SPECIFICITY

IRP-1	5'-AGGTTTGTGGAGGATGACACAG-3'
	5'-AACTTGAAGATGACAGAGATGAG-3'
IRP-2	5'-GCTGATGATGATGATGATGATGATG-3'
	5'-AGCTTATGATGATGATGATGATG-3'
LMP-1	5'-GCTGATGATGATGATGATGATGATG-3'
	5'-ATGCTGATGATGATGATGATGATG-3'
IRP-3, 5'	5'-ATGATGATGATGATGATGATGATG-3'
	5'-ATGATGATGATGATGATGATGATG-3'
IRP-4	5'-ATGATGATGATGATGATGATGATG-3'
	5'-ATGATGATGATGATGATGATGATG-3'
CPNA	5'-ATGATGATGATGATGATGATGATG-3'
IRP-6	5'-ATGATGATGATGATGATGATGATG-3'
	5'-ATGATGATGATGATGATGATGATG-3'
APN-1	5'-ATGATGATGATGATGATGATGATG-3'
	5'-ATGATGATGATGATGATGATGATG-3'
APN-2	5'-ATGATGATGATGATGATGATGATG-3'
IRP-8	5'-ATGATGATGATGATGATGATGATG-3'
	5'-ATGATGATGATGATGATGATGATG-3'

Conclusions

EHF does present as a small subset of breast carcinomas, however, the high PCR positive PC is largely an artifact of lesions collected by lymphatics.

There is a discrepancy between the 2000 and 2011 census.

Acknowledgment

The U.S. Army Medical Research Materiel Command (DAMRC) (Fort Detrick, MD) supported this work.

Table 1. Frequency of EHV-1 isolates from horses and ponies in age to 6 months.

[illegible]
$$N, A, \text{ and } \sigma \text{ are finite, } \beta, \gamma, \text{ and } \tau \text{ are positive reals, } \beta + \gamma + \tau = 1, \text{ and } \beta \neq 0.$$

APPENDIX 2.

The PI's contacting information

Dr. Wing C. Chan, MD
Department of Pathology and Microbiology
University of Nebraska Medical Center,
983135 Nebraska Medical Center
Omaha, NE, 68198-3135

Tel: 402-559-7684
Fax: 402-559-6018
E-mail: jchan@unmc.edu